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Evaluation of the pathogenicity and virulence of three strains of Salmonella organisms in calves and pigs

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Abstract

Objective—To assess in pigs the pathogenicity and virulence of 3 strains of *Salmonella* spp capable of causing atypical salmonellosis in cattle.

Animals—36 Holstein calves and 72 pigs experimentally infected with *Salmonella* spp

Procedures—Representative *Salmonella* strains associated with 3 new disease phenotypes (protozoa-mediated hypervirulence, multisystemic cytopathicity, and encephalopathy) that have been characterized in cattle during the past 10 years were orally inoculated into pigs. Clinical manifestations were compared with those observed in cattle. Samples were collected from various tissues, and the presence of Salmonella organisms was assessed qualitatively and quantitatively by use of *Salmonella*-selective media

Results—Of the 3 unique *Salmonella* disease phenotypes observed in cattle, only protozoa-mediated hypervirulence was observed in pigs. Hypervirulence was related to a more rapid onset of disease and higher pathogen burden in pigs than in cattle. This phenotype was observed in pigs inoculated with multiresistant *Salmonella enterica* serotypes Typhimurium or Choleraesuis bearing the *Salmonella* genomic island 1 (SGI1) integron.

Conclusions and Clinical Relevance—*Salmonella* hypervirulence was identified in pigs noculated with SGI1-bearing strains exposed to free-living protozoa. Additionally, an SGI1-bearing strain of *Salmonella* Choleraesuis was detected that resulted in augmented virulence in pigs. Therefore, it appeared that protozoa-associated salmonellosis was analogous in pigs and cattle. *Salmonella*-mediated encephalopathy and multisystemic cytopathicity did not appear to be relevant diseases in pigs.

Disciplines

Animal Diseases | Veterinary Pathology and Pathobiology

Comments

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During the past 10 years, 3 unique types of salmonellosis in cattle have been identified and characterized by our laboratory group. The first pathotype involved a strain of *Salmonella enterica* serotype Typhimurium in a multifocal outbreak of atypical salmonellosis in veal calves.[1] Affected calves had a polysystemic disease characterized by abomasitis, peritonitis, and polyserositis, and the implicated strains could be isolated from tissues (eg, renal and testicular tissues) not usually associated with *Salmonella* infection. An in vivo technique was developed for this strain, and most of the clinical findings were reproduced.[1] Past outbreaks were characterized by high morbidity and mortality rates,[1] whereas current anecdotal reports of sporadic outbreaks (1 or 2 outbreaks/y) indicate a much lower morbidity.

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Abbreviations

BGS	Brilliant green sulfa
DT104	Phagetype DT104
SGI1	*Salmonella* genomic island 1
TH11	Phagetype TH11

The second pathotype involved *Salmonella* Typhimurium DT104. This strain gained attention in the 1990s as a multiresistant pathogen that had an ampicillin-chloramphenicol-streptomycin-sulfonamide-tetracycline antibiogram. *Salmonella* Typhimurium DT104 apparently has an enhanced ability to cause disease as underscored by a 13-fold increase in mortality rate in cattle, compared with the mortality rate for salmonellosis caused by antimicrobial-susceptible strains.[2] Resistance of *Salmonella* Typhimurium DT104 to multiple antimicrobials is the result of acquiring a mobile DNA integron structure (subsequently designated as SGI1[3]) that contains genes encoding resistance to 5 antimicrobials.[4] More than 30 other genes, none of which contribute to antimicrobial resistance, are also contained in SGI1.[3] A study[5] conducted by our laboratory group implicated rumen protozoa (which are normal flora of the bovine rumen) as an environmental factor causing DT104 hypervirulence. The rumen protozoa–SGI1

interrelationship involves rumen protozoa engulfing DT104 and, with the aid of an SGI1 gene designated as SO13, hyperexpressing cellular invasion genes.[6] The DT104-laden rumen protozoa then are lysed in the abomasum, thus enabling DT104 to safely reach the small intestine where the hyperactivated invasion genes facilitate a rapid progression to systemic salmonellosis.[5] *Salmonella* Typhimurium DT104 continues to be anecdotally reported in field outbreaks of salmonellosis in cattle in which virulence appears to be increased.

The third pathotype involved 3 strains of *Salmonella* organisms capable of causing neurologic disease in cattle recently exposed to stressful situations, such as transportation or commingling. These strains included *S enterica* serotypes Saint-paul, Montevideo, and Enteriditis isolated from calves in Minnesota and Wisconsin.[5] Affected calves had signs of moderate to severe neurologic disease ranging from excessive ear fluttering to seizures, and some affected calves had permanent neurologic deficits. The neurologic effects of these strains were reproduced in a laboratory setting by use of a norepinephrine-based stress-infection technique.[5] These outbreaks of *Salmonella* encephalopathy are now extremely rare, but the potential for zoonotic transmission and the dramatic nature of the disease warrant further investigation in other food-producing animals.

Bovine-adapted *S enterica* serotype Dublin and porcine-adapted *S enterica* serotype Choleraesuis harbor SGI1 (unpublished observations). These findings prompted us to conduct the study reported here and investigate the protozoa-mediated virulence attributes of these strains in their respective hosts. Pigs and cattle were inoculated with protozoa containing various SGI1-bearing *Salmonella* organisms, followed by determinations of the resulting pathogen loads. Because of anecdotal reports suggesting unusual aspects of extraintestinal salmonellosis in pigs, the study reported here also investigated the potential for *Salmonella*-associated cytopathicity and neuropathogenicity in pigs. For cytopathicity studies, a relevant strain of *Salmonella* Typhimurium was inoculated into pigs in which pathogen loads were evaluated in numerous tissues, including those not typically associated with salmonellosis. For neuropathogenicity studies, pigs were inoculated with neuropathogenic *Salmonella* Saint-paul and then monitored for stress-mediated neurologic disease and evidence of *Salmonella* organisms in various tissues.

Materials and Methods

Animals—The in vivo portion of the study involved the use of 1-to 2-week-old male Holstein calves (n = 36) and 10-day-old mixed-breed pigs of both sexes (72). Animal experiments were approved by the Iowa State University Institutional Animal Care and Use Committee.

Bacterial strains and preparation—Bacterial strains used in the study were summarized[5,7–12] (**Appendix 1**). Strain LNWI[9] (designated as *Salmonella* TyphimuriumCYP) served as the cytopathic (ie, CYP) strain, whereas *Salmonella* Typhimurium DT104 strain 98-795[8] was used in experiments involving protozoa. Other SGI1-bearing strains included recently acquired isolates of *Salmonella* Dublin and *Salmonella* Choleraesuis. *Salmonella* Saint-paulNPG was chosen as a representative neuropathogenic (ie, NPG) strain, and SARB strains[12] and certain SGI1-free strains served as control strains. Bacteria were stored in cryopreservation tubes containing 50% glycerol and 50% Lennox L broth[a] at –80°C and were grown in or on Lennox L broth or culture agar[b] prior to use.

Isolation of SGI1-bearing isolates of *Salmonella* Dublin and *Salmonella* Choleraesuis—For both serotypes, approximately 150 to 500 isolates were obtained from clinical and nonclinical isolates submitted to the National Veterinary Services Laboratories from 2004 to 2006. Serotype-specific isolates were pooled and grown aerobically for 16 hours at 37°C in 500 mL of Lennox L broth that contained ampicillin[c] (100 µg/mL) and chloramphenicol[c] (32 µg/mL). One milliliter of flocculent culture was then subcultured aerobically for 16 hours at 37°C in 50 mL of Lennox L broth that contained streptomycin[c] (64 µg/mL) and sulfamethoxazole[c] (512 µg/mL). Individual colonies were isolated by plating 30-µL aliquots on culture agar[b] that contained ampicillin, chloramphenciol, streptomycin, and sulfamethoxazole. The presence of SGI1 was assessed in individual colonies via a PCR assay that used the *floR-tetR* amplicon.[8]

Assessment of other SGI1 genes in isolates of *Salmonella* Dublin and *Salmonella* Choleraesuis—To evaluate the fidelity of SGI1 in isolates that had positive results for *floR-tetR*, 4 other SGI1 genes were examined. These genes were SO13, *tnpR*, *aadA2*, and *pse-1*. The PCR conditions were as described elsewhere[8] for the *floR-tetR* amplicon. The oligonucleotide sequences were summarized[6,8,13] (**Appendix 2**).

***Salmonella* invasion assays following survival within *Acanthamoeba castellanii* or HEp-2 cells**—*Acanthamoeba castellanii*[d] and HEp-2 cells[e] (ie, immortal adherent mammalian epithelial cells that are used for assessing *Salmonella* virulence[10]) were maintained as described elsewhere.[6] Approximately 10^9 bacteria were added to approximately 10^5 *A castellanii* or HEp-2 cells. The *Salmonella*-protozoa mixture was then gently mixed for 16 hours at 37°C in a sealed 5-mL glass tube, whereas the *Salmonella*–HEp-2 mixture was maintained at 37°C in a 5% CO_2 humidified incubator. At the end of the 16-hour incubation period, extracellular *Salmonella* organisms were killed by the addition of florfenicol[f] (300 µg/mL). Then, HEp-2 cells were trypsinized and resuspended in Coleman buffer.[5,6] Cells were lysed by centrifugation (5,000 × *g* for 60 seconds) with 2.5mM glass beads and a mini-bead beater.[g] The lysate was centrifuged at 1,000 × *g* for 2 minutes and then resuspended in 350 µL of Lennox L broth. Of the 350 µL, 25 were used for selective enumeration and 300 were used for an invasion assay performed in triplicate (ie, 100 µL/well), as described elsewhere.[5,6] Percentage invasion was calculated by dividing the number of CFUs recovered by the number of CFUs added.

In vivo infection experiments involving protozoa-laden *Salmonella* organisms—For all in vivo experiments, animals were assigned to groups by use of

a randomization procedure. *Acanthamoeba castellanii* was chosen for use in these experiments because this protozoan can mediate *Salmonella* hypervirulence[6] and is often associated with water and thus is relevant to potable water used in swine operations.

Approximately 10^9 bacteria were added to approximately 10^5 *A castellanii* or HEp-2 cells, and the mixtures were subjected to the same regimen described for the invasion assay. *Salmonella*-laden HEp-2 cells were trypsinized and removed from tissue culture dishes prior to in vivo challenge exposure. Previous studies[5,6] have revealed that approximately 40% to 60% of *Salmonella* organisms are recovered from eukaryotic cells after coincubation.

The *Salmonella*–eukaryotic cell mixture was then placed in a gelatin capsule, and the capsule was orally administered to 1- to 2-week-old male Holstein calves or 10-day-old mixed-breed pigs of both sexes. For each strain of *Salmonella* spp, 6 pigs and 3 calves were inoculated with *A castellanii* cells that contained *Salmonella* spp, whereas 3 animals received HEp-2 cells that contained *Salmonella* spp (the latter was not administered to calves for *Salmonella* Typhimurium DT104 and TH11). All animals received approximately 4.5×10^6 CFUs/kg. Animals were monitored every 8 to 12 hours to evaluate changes in appetite, fecal consistency, and rectal temperature. Bacteriologic examinations were performed on blood samples collected every 12 hours.

At 36 to 96 hours after inoculation, animals were euthanatized. Calves were premedicated by administration of xylazine hydrochloride[h] (0.45 mg/kg, IM), which was followed by administration of pentobarbital[i] (1 mg/kg, IV). Pigs were euthanatized by administration of pentobarbital (1 mg/kg, IP). The spleen was then removed from each animal and submitted for bacteriologic examinations.

In vivo infection experiments involving cytopathic strains—Animals were challenge-exposed via oral administration of 4.5×10^6 CFUs of *Salmonella* TyphimuriumCYP/kg; these *Salmonella* organisms were grown aerobically for 16 hours at 37°C. Six pigs and 3 calves were inoculated with *Salmonella* TyphimuriumCYP, and 6 pigs and 3 calves were inoculated with the control strain (*Salmonella* TyphimuriumCYP-SlyA', which is not capable of cytopathic effects[11]).

When pronounced clinical signs, such as diarrhea, dehydration, and pyrexia (typically at 2 to 4 days after inoculation), were observed, affected animals were immediately euthanatized. Euthanasia was performed with xylazine and pentobarbital as described previously. Various samples were collected, including samples of the intestines, blood, spleen, lungs, liver, kidneys, and gonads; the kidneys and gonads were collected because these 2 tissues rarely harbor *Salmonella* spp, except for the novel cytopathic strains.[1,10,11]

In vivo infection-stress experiments involving neuropathogenic strains—Animals were challenge-exposed via oral administration of 4.5×10^6 CFUs of *Salmonella* Saint-paulNPG/kg; these *Salmonella* organisms were grown aerobically for 16 hours at 37°C. For each strain, 3 pigs and 3 calves received daily doses of norepinephrine[c] (45 µg/kg, IM) starting on the day of inoculation and continuing until the animals were euthanatized. As a control treatment, 3 calves and 3 pigs were challenge-exposed by oral administration of *Salmonella* Saint-paulNPG and administered a placebo (volume of saline [0.9% NaCl] solution identical to the volume of norepinephrine, IM). Additionally, 3 calves and 3 pigs were challenge-exposed by oral administration of *Salmonella* Saint-paulNPG and administered daily doses of dexamethasone[j] (0.1 mg/kg, IM). The control strain, which was administered to 3 calves and 3 pigs, was *Salmonella* Saint-paulSARB, which is not capable of causing neurologic disease in cattle.[7]

Animals were monitored for neurologic disease (seizures or clinical signs such as excessive ear fluttering, ataxia, opisthotonus, and proprioceptive placing deficits). When signs of neurologic disease were observed (typically 4 to 9 days after inoculation), affected animals were immediately euthanatized. An animal with neurologic disease was defined as an animal that had convulsions or that had at least 2 of the aforementioned 4 clinical signs of neurologic disease. Animals that did not have signs of neurologic disease were euthanatized 12 to 14 days after inoculation (ie, at least 3 or 4 days after animals with signs of neurologic disease were euthanatized). Animals were euthanatized with xylazine and pentobarbital, as described previously. Samples of the intestines, blood, spleen, and brain were collected from each animal.

Bacteriologic-based detection of *Salmonella* spp in various tissues—For qualitative assessment of *Salmonella* spp, microbes were selectively cultured by inoculation of 3 to 5 g of each sample into 100 mL of GN Hajna broth[k] and incubation overnight at 37°C in aerobic conditions. Then, 100 µL of the inoculum was transferred into 5 mL of Rappaport-Vassiliadis R10 broth,[k] which was also incubated overnight at 37°C in aerobic conditions. Cultures were then transferred to plates containing BGS agar[k]; plates were incubated overnight at 37°C. Individual colonies recovered from selective plates were grown overnight in Lennox L broth and identified by use of a *Salmonella*-specific PCR assay targeting the sipB-sipC junction.[8] All media included antimicrobials specific to the antibiogram of the individual isolate.

For quantitation of *Salmonella* organisms in blood and spleen tissues as an estimate of systemic pathogen load and as an indirect correlate of clinical signs, samples were directly inoculated on plates containing BGS agar. For blood samples, 100 µL was inoculated onto each of 10 separate plates. For spleen samples, 50 to 75 g of sample was homogenized, filtered with cheesecloth, and then inoculated onto 10 plates containing BGS agar (ie, approx 3 to 5 g of sample/plate). Colonies were enumerated the following day. Because these tissues can harbor similar numbers of bacteria at early time points in the infectious process, CFU data for spleen samples were pooled with counts from blood samples to provide the final data of each time course experiment. The identity of *Salmonella* strains was confirmed by use of antisera-agglutination–based serogrouping[l] in addition to confirmation of the original antibiogram for each isolate.

Statistical analysis—Statistical analysis was performed by use of an ANOVA with the Scheffe *F* test for multiple comparisons. Analyses were performed by use of a commercially available statistical program.[m] Although multiple samples were obtained from each tissue, each tissue-associated value represented a mean for the replicates from that particular tissue.

Results

Isolation and preliminary characterization of SGI1-bearing *Salmonella* Choleraesuis and *Salmonella* Dublin—From the pools of *Salmonella* Choleraesuis and *Salmonella* Dublin isolates, 3 colonies of *Salmonella* Choleraesuis and 2 colonies of *Salmonella* Dublin had ampicillin-chloramphenicol-streptomycin-sulfonamide antibiograms. One colony of each serotype yielded the *floR-tetR* amplicon (**Figure 1**); this amplicon is present in SGI1.[8]

Profile of SGI1 genes in isolates of *Salmonella* Choleraesuis and *Salmonella* Dublin with the *floR-tetR* amplicon—To evaluate the nature of SGI1 in isolates with the *floR-tetR* amplicon, 4 other SGI1 genes (SO13, *tnpR*, *aadA2*, and *pse-1*) were evaluated. The function of the SO13 gene is unknown,[3] although a recent study[6] revealed that it participates in upregulating invasion genes (and thus virulence) in *Salmonella* Typhimurium DT104 and other strains containing SGI1.[6] The *tnpR* gene is a transposase-encoding gene present in many integrons,[14] whereas *aadA2* and *pse-1* confer resistance to streptomycin-spectinomycin and ampicillin-amoxicillin, respectively. All 5 genes were detected in both *Salmonella* Choleraesuis and *Salmonella* Dublin.

Protozoa-dependent invasion assays for SGI1-bearing *Salmonella* Choleraesuis and *Salmonella* Dublin—To determine whether the SGI1-bearing strains of *Salmonella* Choleraesuis and *Salmonella* Dublin are hyperinvasive following exposure to protozoa (as has been reported[5] for *Salmonella* Typhimurium DT104), bacteria were incubated with free-living protozoa (*A castellanii* cells) and invasion assays were performed. As a control sample, bacteria were incubated with HEp-2 cells. Bacteria were recovered from protozoa or HEp-2 cells and then subjected to a standard tissue culture invasion assay by use of HEp-2 cells.[10] Protozoa were capable of augmenting invasion for the SGI1-bearing strains of *Salmonella* Choleraesuis and *Salmonella* Dublin (**Figure 2**). The 6- to 7-fold increase was similar to that observed for *Salmonella* Typhimurium DT104, which parallels that reported[5] for rumen protozoa–mediated hyperinvasion and hypervirulence. No such effect was observed for *Salmonella* Typhimurium TH11 (a DT104 strain that lacks SGI1[8]) or for the SGI1-free SARB strains of *Salmonella* Choleraesuis and *Salmonella* Dublin.[12]

In vivo experiments to assess protozoa-associated virulence for *Salmonella* Typhimurium DT104 in pigs and cattle—*Salmonella* organisms were incubated with protozoa and then inoculated into pigs and calves to determine whether protozoa-associated *Salmonella* Typhimurium DT104 hypervirulence in calves could extend to pigs. As a control sample, the same *Salmonella* strains were incubated with HEp-2 cells prior to in vivo inoculation.

Protozoa were able to enhance the systemic virulence of *Salmonella* Typhimurium DT104 in pigs (**Figure 3**). This effect was not as dramatic as that observed in calves, as indicated by the results for the present study as well as results of another study.[5] All *Salmonella* Typhimurium DT104–inoculated calves were euthanized by 48 hours after inoculation because of severe clinical manifestations of salmonellosis (eg, pyrexia, diarrhea, lethargy, anorexia, and dehydration). Clinical signs were mild and transient in the *Salmonella* Typhimurium TH11–inoculated calves and in all pigs; thus, these animals were not euthanized until 96

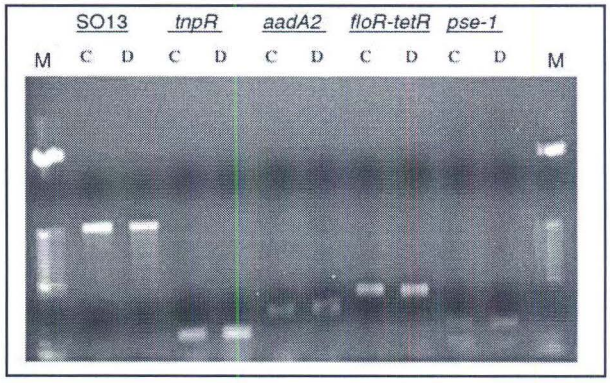

Figure 1—Bands for PCR-based characterization of SGI1 in *Salmonella enterica* serotype Choleraesuis (C) and *S enterica* serotype Dublin (D). Genes evaluated were SO13[3,6], *tnpR* (resolvase), *aadA2* (spectinomycin-streptomycin resistance), *floR-tetR* (junction between *floR* [florfenicol resistance] and *tetR* [regulator of tetracycline resistance]), and *pse-1* (carbenicillinase). Predicted amplicon sizes are 800 bp (SO13), 172 bp (*tnpR*), 250 bp (*aadA2*), 280 bp (*floR-tetR*), and 231 bp (*pse-1*). M = DNA ladder.

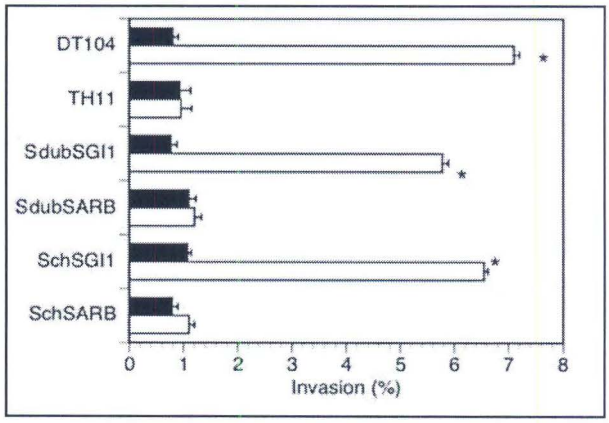

Figure 2—Protozoa-mediated effects on the invasion of HEp-2 cells (black bars) and *Acanthamoeba castellanii* cells (white bars) by SGI1-bearing isolates of *Salmonella* Choleraesuis (Sch) and *Salmonella* Dublin (Sdub). Mean ± SEM values for invasion percentage were determined after recovery from HEp-2 or *A castellanii* cells. Control isolates included SGI1-bearing *S enterica* serotype Typhimurium DT104, *Salmonella* Typhimurium TH11 (ie, SGI1-free *Salmonella* Typhimurium DT104), and *Salmonella* CholeraesuisSARB and *Salmonella* DublinSARB (both of which lack SGI1). Invasion percentage was calculated as follows: 100 X (CFUs recovered from the HEp-2 cells/CFUs added to the HEp-2 cells). *Within an isolate, value differs significantly ($P < 0.05$) from the value for the HEp-2 cells.

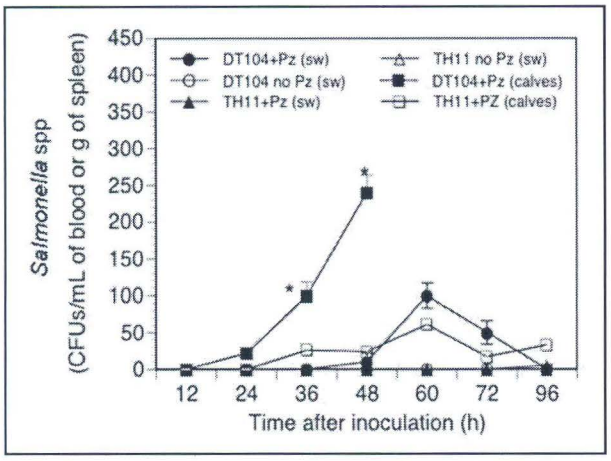

Figure 3—Evaluation of the protozoal-mediated effects on time-dependent systemic infectivity of *Salmonella* Typhimurium DT104 in pigs (SW) or calves. Data represent the mean ± SEM number of CFUs of *Salmonella* Typhimurium DT104 or *Salmonella* Typhimurium TH11 (ie, the SGI1-free version of *Salmonella* Typhimurium DT104) recovered from calves or pigs inoculated with protozoa (Pz) that contained *Salmonella* spp or with HEp-2 cells (no Pz) that contained *Salmonella* spp; values were calculated for 3 or 6 samples, each of which was assayed in triplicate. The last data points (48 hours after inoculation for *Salmonella* Typhimurium DT104–inoculated calves and 96 hours after inoculation for pigs and *Salmonella* Typhimurium TH11–infected calves) represent cumulative data for samples of blood and spleen. *Within a time point, value differs significantly ($P < 0.05$) from the relevant control treatment (eg, *Salmonella* Typhimurium DT104 vs *Salmonella* Typhimurium TH11 or Pz vs no Pz).

hours after inoculation. Protozoa-associated *Salmonella* Typhimurium DT104 were recovered to a significantly greater extent than were *Salmonella* Typhimurium DT104 not exposed to protozoa. The SGI1-free strains were not recovered from samples of blood or spleen obtained from inoculated pigs, regardless of protozoa exposure. *Salmonella* organisms were not recovered from most of the spleen tissues obtained from animals challenge-exposed with SGI1 free strains or strains that were not exposed to protozoa.

In vivo experiments to assess protozoa-associated virulence for SGI1-bearing host-adapted *Salmonella* spp in pigs and cattle—In vivo experiments were conducted to determine whether protozoa-associated hypervirulence could be detected in 2 new SGI1-bearing strains of *Salmonella* spp adapted to pigs (*Salmonella* Choleraesuis) and cattle (*Salmonella* Dublin). *Salmonella* organisms were incubated with protozoa and then inoculated into calves and pigs. As a control sample, the same *Salmonella* strains were incubated with HEp-2 cells prior to in vivo inoculation. Control strains included the SARB versions of each serotype.

Protozoa were able to enhance the systemic virulence of SGI1-bearing *Salmonella* Choleraesuis in pigs (**Figure 4**). This effect was evident at an earlier onset of detectable pathogen load (36 hours for *Salmonella* CholeraesuisSGI plus protozoa and 48 hours for all others) and an earlier onset of pathogen burden necessitating euthanasia (48 hours for *Salmonella* CholeraesuisSGI plus protozoa and 60 hours for all others). Protozoa were incapable of augmenting the virulence of SGI1-bearing *Salmonella* Dublin.

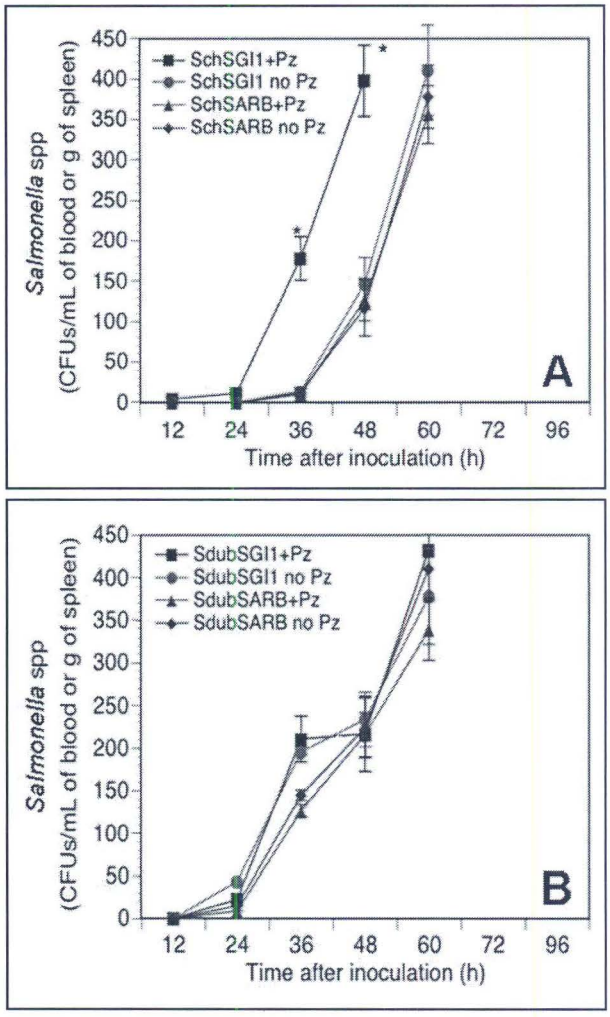

Figure 4—Protozoal-mediated effects on time-dependent systemic infectivity of SGI1-bearing host-adapted *Salmonella* spp in pigs (A) or calves (B). Data represent mean ± SEM number of CFUs of *Salmonella* organisms recovered from pigs or calves inoculated with protozoa (Pz) that contained *Salmonella* spp or with HEp-2 cells (no Pz) bearing *Salmonella* spp; values were calculated for 3 samples, each of which was assayed in triplicate. *See* Figure 3 for remainder of key.

In vivo experiments to evaluate the cytopathicity of strain *Salmonella* TyphimuriumCYP in pigs—In vivo experiments were conducted to determine whether strain *Salmonella* TyphimuriumCYP could evoke cytopathic effects and polysystemic salmonellosis in pigs, similar to that observed in veal calves.[10] *Salmonella* TyphimuriumCYP was inoculated into young calves and pigs. As a control sample, animals were inoculated with *Salmonella* TyphimuriumCYP-SlyA', which is not capable of cytopathic activity.[11] As an additional control sample, tissue samples were obtained from animals inoculated with the host-adapted serotypes.

Pigs did not harbor *Salmonella* TyphimuriumCYP in any of the extraintestinal sites (**Table 1**). In contrast, *Salmonella* TyphimuriumCYP was recovered from all tissues obtained from the calves, including organs not usually associated with salmonellosis (eg, kidneys

Table 1—Qualitative assessment of *Salmonella* organisms recovered* from tissues of male Holstein calves and male and female mixed-breed pigs inoculated with *Salmonella enterica* serotype TyphimuriumCYP, compared with recovery for the control treatment noncytopathic *Salmonella* TyphimuriumCYP-SlyA' and the host-adapted strains of *S enterica* serotype DublinSARB in calves and *S enterica* serotype CholerasuisSARB in pigs.

	Calves							Pigs						
Strain	**In**	**Bl**	**Sp**	**Lu**	**Li**	**Ki**	**Go**	**In**	**Bl**	**Sp**	**Lu**	**Li**	**Ki**	**Go**
Salmonella TyphimuriumCYP	A	A	A	A	A	A	A	A	NI	NI	NI	NI	NI	NI
Salmonella TyphimuriumCYP-SlyA'	A	NI	C	NI	NI	NI	NI	A	NI	NI	NI	NI	NI	NI
Salmonella DublinSARB†	A	A	A	C	NI	NI	NI	—	—	—	—	—	—	—
Salmonella CholeraesuisSARB†	—	—	—	—	—	—	—	A	A	A	B	NI	NI	NI

**Salmonella* organisms were isolated from all 3 inoculated animals (A), 2 of 3 inoculated animals (B), or 1 of 3 inoculated animals (C) or was not isolated (NI). †Infected with the SARB strains in HEp-2 cells.
— = Not applicable. Bl = Blood. Go = Gonads. In = Intestines. Ki = Kidneys. Li = Liver. Lu = Lungs. Sp = Spleen.

Table 2—Qualitative assessment of *Salmonella* organisms recovered from male Holstein calves and male and female mixed-breed pigs inoculated with *S enterica* serotype Saint-paulNPG or nonneuropathogenic *Salmonella* Saint-paulSARB and injected with norepinephrine or dexamethasone to simulate stress.

		Calves				Pigs			
		Tissue source				Tissue source			
Strain	**Treatment**	**In**	**Bl-Sp**	**Br**	**Neurologic disease***	**In**	**Bl-Sp**	**Br**	**Neurologic disease***
Salmonella Saint-paulNPG	Saline (0.9% NaCl) solution	A	NI	NI	A	A	NI	NI	NI
	Norepinephrine	A	A	A	A	A	NI	NI	NI
	Dexamethasone	A	B	B	B	A	NI	NI	NI
Salmonella Saint-paulSARB	Norepinephrine	A	NI	NI	NI	A	NI	NI	NI

*Neurologic disease was defined as animals that had convulsions or that had at least 2 of 4 clinical signs of neurologic disease (excessive ear fluttering, ataxia, opisthotonus, and proprioceptive placing deficits).
A = Isolated or observed in all 3 animals. B = Isolated or observed in 2 of 3 animals. Bl-Sp = Blood and spleen combined. Br = Brain. In = Intestines. NI = Not isolated or identified.

and testes). The noncytopathic strain *Salmonella* TyphimuriumCYP-SlyA' was recovered from all intestinal samples and from the spleen of 1 calf. Both host-adapted serotypes were recovered from extraintestinal sites (ie, blood, spleen, and lungs) usually associated with these pathogens in their preferred hosts. Renal and gonadal tissues did not yield either host-adapted strain from the respective host.

In vivo experiments to evaluate the neuropathogenic effects of *Salmonella* Saint-paulNPG in pigs—In vivo experiments were conducted to determine whether strain *Salmonella* Saint-paulNPG could lead to neurologic disease in pigs, similar to that observed in stressed calves.[7] *Salmonella* Saint-paulNPG was inoculated into young calves and pigs, and these animals were also administered consecutive daily doses of norepinephrine or dexamethasone to mimic stress. As a control sample, animals were inoculated with *Salmonella* Saint-paulSARB, which is not capable of eliciting signs of neurologic disease in calves.[7]

Pigs did not have signs of clinical disease, and *Salmonella* organisms could only be recovered from the intestines (**Table 2**). In contrast, calves had signs of neurologic disease when inoculated with *Salmonella* Saint-paulNPG and administered norepinephrine or dexamethasone. *Salmonella* organisms were isolated from the intestines, blood, spleen, and brain of calves with signs of neurologic disease. Nonneuropathogenic *Salmonella* Saint-paulSARB was only recovered from the intestines of calves, similar to results in another study.[7]

Discussion

Several unique strains of *Salmonella* spp have unusual virulence and pathogenicity properties in calves. However, these effects have not been confirmed clinically in pigs. Hypervirulence in cattle is attributable to the presence of both SGI1 and survival within protozoa, whereas cytopathogenicity and neuropathogenicity have been associated with specific strains of *Salmonella* spp under certain conditions. Stress has been implicated in the etiology of the encephalopathies related to the neuropathogenic *Salmonella* spp.

Analysis of results for the study reported here indicated that protozoa-associated hypervirulence of *Salmonella* Typhimurium DT104 can be observed in pigs, although the effect is not as pronounced as that found in cattle. In pigs, *Salmonella* Typhimurium DT104 infection peaked at 60 hours after inoculation but decreased with time, whereas the pathogen increased in a time-dependent logarithmic manner in cattle until clinical signs warranted euthanasia at 48 hours. The SGI1-bearing *Salmonella* Choleraesuis had enhanced virulence following exposure to protozoa, but this effect was only related to the onset of disease. *Salmonella* Dublin associated with protozoa did not have significant enhancement of virulence in cattle, and this may have been related to its inherent putative maximal degree of virulence in calves.

Further comparisons revealed that *Salmonella* TyphimuriumCYP could not evoke cytopathic effects and polysystemic salmonellosis in pigs similar to those observed in cattle. *Salmonella* Saint-PaulNPG caused

neurologic disease in cattle, but similar effects were not observed in pigs. These 2 findings are not altogether surprising because the *Salmonella*-associated cytopathogenicity and neuropathogenicity appear to be dependent on neurohormonal factors in cattle that may be divergent from those in swine.

Overall, these *Salmonella* strains typically are more virulent in cattle. For SGI1-bearing *Salmonella* spp, protozoa-associated hyperinvasion led to augmented virulence in pigs, and this was evident in the onset of disease for *Salmonella* Choleraesuis. From a clinical perspective, the hypervirulence phenomenon has not been reported in pigs because these animals have a lower exposure to protozoa than do ruminants (ie, protozoa-mediated hypervirulence of *Salmonella* spp can occur in pigs, but the incidence is probably not as high as that in cattle). In contrast, cytopathic and neuropathogenic strains were not capable of expressing their respective phenotypes in pigs.

a. Lennox broth base, 12780-052, Invitrogen, Carlsbad, Calif.
b. Lennox agar, 22700-025, Invitrogen, Carlsbad, Calif.
c. Sigma Chemical Co, St Louis, Mo.
d. 30010, ATCC, Manassas, Va.
e. CCL-23, ATCC, Manassas, Va.
f. NuFlor, Schering-Plough, Kenilworth, NJ.
g. Biospec Products, Bartlesville, Okla.
h. Phoenix Scientific Inc, St Joseph, Mo.
i. Fort Dodge Animal Health, Fort Dodge, Iowa.
j. Vedco, St Joseph, Mo.
k. Becton-Dickinson, Sparks, Md.
l. Difco, Becton-Dickinson, Sparks, Md.
m. Statview, SAS Institute Inc, Cary, NC.

References

1. Carlson SA, Stoffregen WC, Bolin SR. Abomasitis due to multiple antibiotic resistant *Salmonella enterica* serotype Typhimurium phagetype DT104. *Vet Microbiol* 2002;85:233–240.
2. Wall PG, Morgan D, Lamden K, et al. A case control study of infection with an epidemic strain of multiresistant *Salmonella typhimurium* DT104 in England and Wales. *Commun Dis Rep CDR Rev* 1994;4:R130–R135.
3. Boyd D, Peters G, Cloeckaert A, et al. Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *J Bacteriol* 2001;183:5725–5732.
4. Briggs CE, Fratamico PM. Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. *Antimicrob Agents Chemother* 1999;43:846–849.
5. Rasmussen MA, Carlson SA, Franklin SK, et al. Exposure to rumen protozoa leads to enhancement of invasion and pathogenicity for multiple antibiotic resistant *Salmonella* bearing SGI-1. *Infect Immun* 2005;73:4668–4675.
6. Carlson SA, Sharma VK, McCuddin ZP, et al. Involvement of a *Salmonella* genomic island 1 gene in the rumen protozoan-mediated enhancement of invasion for multiple-antibiotic-resistant *Salmonella enterica* serovar Typhimurium. *Infect Immun* 2007;75:792–800.
7. McCuddin ZP, Carlson SA, Sharma VK. Experimental reproduction of bovine *Salmonella* encephalopathy using a norepinephrine-based stress model. *Vet J* 2008;175:82–88.
8. Carlson SA, Bolton LF, Briggs CE, et al. Detection of *Salmonella typhimurium* DT104 using multiplex and fluorogenic PCR. *Mol Cell Probes* 1999;13:213–222.
9. Carlson S, Meyerholz D, Stabel T, et al. Secretion of a putative cytotoxin in multiple antibiotic resistant *Salmonella enterica* serotype Typhimurium phagetype DT104. *Microb Pathog* 2002;31:201–204.
10. Wu MT, Carlson SA, Meyerholz DK. Cytopathic effects observed upon expression of a repressed collagenase gene present in *Salmonella* and related pathogens: mimicry of a cytotoxin from multiple antibiotic-resistant *Salmonella enterica* serotype Typhimurium phagetype DT104. *Microb Pathog* 2002;32:279–287.
11. Carlson SA, McCuddin ZP, Wu MT, et al. SlyA partially regulates the collagenase-mediated cytopathic phenotype specific for multiresistant *Salmonella*. *Microb Pathog* 2005;38:181–187.
12. Pabbaraju K, Miller W, Sanderson K. Distribution of intervening sequences in the genes for 23S rRNA and rRNA fragmentation among strains of *Salmonella* Reference Collection B (SARB) and SARC sets. *J Bacteriol* 2000;182:1923–1929.
13. Frana TS, Carlson SA, Griffith RW. Relative distribution and conservation of genes encoding aminoglycoside-modifying enzymes in *Salmonella enterica* serotype typhimurium phage type DT104. *Appl Environ Microbiol* 2001;67:445–448.
14. Guerra B, Soto S, Helmuth R, et al. Characterization of a self-transferable plasmid from *Salmonella enterica* serotype typhimurium clinical isolates carrying two integron-borne gene cassettes together with virulence and drug resistance genes. *Antimicrob Agents Chemother* 2002;46:2977–2981.

Appendix 1

Salmonella enterica strains and plasmids used in the study.

Strain designation	SGI1 status	Virulence phenotype	Additional information	References
Salmonella Typhimurium DT104	+	Hyperinvasive after exposure to protozoa	Strain 98-795	5
Salmonella Typhimurium TH11	—	ND	Serotype Typhimurium DT104	8
Salmonella TyphimuriumCYP	+	Cytopathic in veal calves	Serotype Typhimurium DT104 (formerly designated as strain LNWI)	9, 10
Salmonella TyphimuriumCYP-SlyA'	+	ND	Strain LNWI bearing a truncation in the *slyA* gene; noncytopathic	11
Salmonella DublinSARB	—	Adapted to cattle; nonneuropathogenic	NN	7, 12
Salmonella CholeraesuisSARB	—	Adapted to swine	NN	12
Salmonella DublinSGI1	+	Adapted to cattle	NN	NA
Salmonella CholeraesuisSGI1	+	Adapted to swine	NN	NA
Salmonella Saint-paulNPG	—	Neuropathogenic in stressed calves	NN	7
Salmonella Saint-paulSARB	—	Nonneuropathogenic	NN	7, 12

+ = SGI1 present. — = SGI1 absent. NA = Not applicable; developed for the study reported here. ND = Not determined. NN = Nothing noteworthy.

Appendix 2

The SGI1-specific oligonucleotides and resulting amplicons used in the study for the characterization of multiresistant *S enterica* serotypes Choleraesuis and Dublin.

Oligonucleotide designation	DNA sequence (5′ to 3′)	Amplicon size (bp)	Reference
S013	Forward: ATGAAAATGAATATGTCAACTTCC	800	6
	Reverse: TCATTGGCCTTCCTAAAATAGCAA		6
tnp	Forward: GCACTGTTCGTTTCAATCTGT	172	NA
	Reverse: TGGGAAGAATGCCGCTAGAC		NA
pse-1	Forward: TTTGGTTCCGCGCTATCTG	231	8
	Reverse: TACTCCGAGCACCAAATCCG		8
aadA2	Forward: CGGTGACCATCGAAATTTCG	250	13
	Reverse: CTATAGCGCGGAGCGTCTCGC		13
floR-tetR	Forward: CGCTCCTTCGATCCCGT	280	8
	Reverse: GCTGCGTTCATCTACAACAGAT		8

NA = Not applicable; developed for the study reported here.